

## DESCRIPTION

D&B, B' IMMUNOASSAY FOR BNP

## 5 TECHNYCAL FIELD

The present invention relates to an immunoassay for the brain natriuretic peptide (BNP) which is a member of natriuretic peptide family, more specifically, it relates to an immunoassay for  $\gamma$ -BNP and derivatives thereof.

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## BACKGROUND ART

Natriuretic peptide family includes three members, i.e., atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and type C natriuretic peptide (CNP). Among them, ANP and BNP are cardiac hormones which are mainly biosynthesized in and secreted from the heart. ANP and BNP are similar in structure. ANP is a peptide of 28 amino acids with a ring (circular) structure formed by a disulfide bond between the 7th and the 23rd cysteine residues, while BNP is a peptide of 32 amino acids with a ring structure formed by a disulfide bond between the 10th and the 26th cysteine residues. These mature peptides of 28 and 32 amino acids have been considered to be produced from respective precursor when a leader sequence is cleaved off intracellularly or at the time of secretion. That is, there has been reported that human BNP is first synthesized as a preprohormone (hereinafter, referred to as prepro-BNP) in myocardial cells, which is split before or at the time of secretion between Ser<sup>26</sup>-His<sup>27</sup> to give pro-BNP (hereinafter, referred to as  $\gamma$ -BNP), and which is further split between Arg<sup>102</sup>-Ser<sup>103</sup> to give BNP-32 (hereinafter, referred to as  $\alpha$ -BNP) and BNP(1-76), and that the former exhibits the activity. It

has been considered that at least the ring structure must be remained for the expression of activity.

The secretion of cardiac hormones being stimulated by various heart diseases, it well reflects the change in the cardiac functions. The secretion of ANP is accelerated mainly when the atrium undergoes a load, while the biosynthesis and secretion of BNP are stimulated when the ventricle undergoes a load. Accordingly, both ANP and BNP are useful as indicators in the diagnosis of heart disease. As the progress of investigation in the *in vivo* role of respective hormone, the advantageous features of BNP as an indicator for diagnosing heart disease have become clear. For example, the blood concentration of BNP is only 1/6 of ANP in a normal subject but it becomes higher than ANP in patients of heart failure or the like; the blood concentration of BNP increases in the case of heart failure like ANP, and the plasma concentration of BNP often exceeds that of ANP reflecting more accurately the severity of heart dysfunction; the plasma concentration of both ANP and BNP elevates in peripheral blood and elevation rate is higher in BNP. Moreover, BNP level in patients of heart failure sometimes increases to several tens times to several hundreds times of that of healthy normal subjects, and the change of BNP in the cases of heart failure is so marked that no other hormones are incomparable therewith. For these reasons, the usefulness of BNP measurement has been suggested (Y. Saito et al., Mebio, 12(5), 28, 1995).

Under the conditions, an immunoassay which utilizes anti-BNP antibody and is applicable to the diagnosis of cardiac insufficiency has been proposed. Japanese Patent Publication (KOHYO) 7-507210 describes a method of measuring  $\gamma$ -BNP (1-76) produced by biodegradation by protease or the like. However, this

method is directed to  $\gamma$ -BNP (1-76) which lacks the portion(s) essential for the expression of activity such as ring structure and, therefore, cannot determine the hormone activity directly.

An assay kit for the measurement of  $\alpha$ -BNP having natriuretic activity has been marketed ("BNP-32", Peninsula). With this kit, degradation products of  $\alpha$ -BNP in blood including fragments lacking activity due to the deletion of C-terminal region can also be measured. Taking the low blood concentration of BNP into consideration, the measurements involving the degradation products cannot be disregarded. Accordingly, the said method connotes disadvantages to be an assay for BNP in the establishment of an accurate diagnosis of heart failure.

As a kit for the measurement of BNP free from the disadvantages above has been marketed ("SHIONORIA BNP", Shionogi), which characteristically uses an antibody recognizing the structure essential for the expression of activity. However, this method would be affected significantly by the process for collecting and storing blood sample, because  $\alpha$ -BNP is extremely unstable in collected blood. It is, therefore, suggested that the sample should be specifically treated by, for instance, adding an agent for inhibiting degradation into a blood collecting tube or maintaining the sample at low temperature so as to obtain reliable data. Such procedures may hamper the extensive clinical application of the said BNP assay kit.

## 25 DISCLOSURE OF INVENTION

The present inventors have conducted research intensively for the purpose of establishing an accurate method of diagnosing cardiac diseases involving BNP and found that BNP exists in blood in the form of  $\gamma$ -BNP or its degradation product which at least

comprises structurally the  $\alpha$ -BNP moiety (hereinafter, they are referred to as " $\gamma$ -BNP derivative"), and not in the form of  $\alpha$ -BNP which has so far been considered to be dominant. The inventors have also found that  $\gamma$ -BNP is more stable than  $\alpha$ -BNP in blood, that  
5 is, one role of the N-terminal structure of  $\gamma$ -BNP, among many, would be the stabilization of BNP. The above indicates that an organism biosynthesizes at least 2 kinds of BNP molecule which share the BNP activity but differ in half-life. These findings led the present inventors to have a view that it is indispensable to establish a method  
10 specific for not only  $\alpha$ -BNP but also  $\gamma$ -BNP to accomplish an accurate diagnosis of cardiac diseases.

The present invention provides an immunoassay specific for mammalian  $\gamma$ -BNP derivatives, characterized in that it uses the first antibody which is reactive with mammalian  $\alpha$ -BNP and the second antibody which is reactive with mammalian prepro-BNP or  $\gamma$ -BNP derivatives and not reactive with  $\alpha$ -BNP.  
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As used herein, the term "mammalian  $\alpha$ -BNP" refers to a peptide of low molecular weight having natriuretic activity which is derived from mammalian prepro-BNP or  $\gamma$ -BNP through the removal of N-terminal region as a result of processing at the carboxy terminus of processing signal sequence. In case of human BNP,  $\alpha$ -BNP is a peptide consisting of C-terminal 32 amino acids (Nos. 103-134) of the amino acid sequence of SEQ ID NO: 1 and having a ring structure. The carboxy terminus of processing signal sequence on the prepro-BNP molecule varies slightly depending on the species.  
20  
25 For example, it is No. 102 Arg in case of human BNP while it is No. 100 amino acid in case of porcine or canine BNP.

As used herein, the term "mammalian  $\gamma$ -BNP" refers to a pro-BNP comprising a partial peptide of 32 amino acids corresponding

to  $\alpha$ -BNP at the carboxy terminal region. In case of human  $\gamma$ -BNP, it is pro-BNP of 108 amino acids from No. 27 His to No. 134 His of the amino acid sequence of SEQ ID NO: 1. The term "prepro-BNP" refers to a peptide of 134 amino acids from No. 1 Met to No. 134 His of the 5 amino acid sequence of SEQ ID NO: 1 in case of human.

As used herein, the term "mammalian  $\gamma$ -BNP derivative" refers to a peptide fragment derived from mammalian prepro-BNP or  $\gamma$ -BNP through mainly the *in vivo* protease reaction, which fragment includes or is larger than  $\alpha$ -BNP. Although  $\gamma$ -BNP derivative 10 would comprise a molecule of the same or smaller size compared to  $\gamma$ -BNP in general, it may comprise a molecule larger than  $\gamma$ -BNP. Otherwise specifically mentioned, as used herein, the term " $\gamma$ -BNP derivative" includes  $\gamma$ -BNP itself.

The term "stable", when used herein in connection with BNP, 15 means that a BNP molecule maintains the C-terminal ring structure including C-terminus of BNP and the natriuretic activity after undergoing the degradation by protease, and that the said activity is not significantly decreased even 24 hours from the collection of blood samples. In light of this definition, the  $\gamma$ -BNP derivative as the 20 target substance (analyte) of the present immunoassay is stable.

On the other hand, the term "unstable" means that a BNP sample undergoes degeneration by protease at the C-terminal region and that the natriuretic activity is significantly decreases 24 hours from the collection of blood samples. In light of this definition,  $\alpha$ - 25 BNP is unstable.

#### BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 is a chromatogram obtained in an  $\alpha$ -BNP assay system wherein gel filtration HPLC was conducted using Superdex 75 in a

plasma sample. In Fig. 1, A indicates the position of elution of  $\alpha$ -BNP.

Fig.2 is a chromatogram obtained in an  $\alpha$ -BNP assay system wherein gel filtration HPLC was conducted using Superdex 75 in a plasma sample different from that shown in Fig. 1. In Fig. 2, A indicates the position of elution of  $\alpha$ -BNP.

Fig. 3 is a chromatogram obtained in an immunoassay specific for  $\gamma$ -BNP wherein gel filtration HPLC was conducted using Superdex 75 in a plasma sample same as those shown in Fig. 2. In Fig. 3, A indicates the position of elution of  $\alpha$ -BNP.

Fig.4 is a graph showing the relationships between the storing time and BNP immunoreactivity of  $\gamma$ -BNP kept in human plasma at 25°C.

Fig.5 is a graph showing the relationships between the storing time and BNP immunoreactivity of  $\alpha$ -BNP kept in human plasma at 4°C.

#### BEST MODE FOR CARRING OUT THE PRESENT INVENTION

In one embodiment of the present invention, it is related to a method which uses two antibodies, wherein the first antibody is reactive with mammalian  $\alpha$ -BNP and the second antibody is reactive with prepro-BNP or  $\gamma$ -BNP derivatives and is not reactive with  $\alpha$ -BNP.

Antibodies used in the present method can be monoclonal or polyclonal antibodies. The first antibody can be prepared according to a method known in the art using as an antigen human  $\alpha$ -BNP which is commercially available or chemically synthesized, or a partial peptide thereof. Alternatively, a monoclonal antibody appended to a commercially available  $\alpha$ -BNP assay system (kit) for measuring  $\alpha$ -

BNP ("SHIONORIA", Shionogi) is also available, which is reactive with the C-terminal region of  $\alpha$ -BNP.

As the second antibody, any antibody can be used subject that it meets the conditions above. Preferred examples of such 5 antibody include those specific for the amino acid sequence shown by the amino acid Nos. 27-102 of SEQ ID NO: 1 or metabolites thereof.

The  $\gamma$ -BNP derivatives as an analyte to be measured by the present method preferably include at least the partial amino acid sequence shown by the amino acid Nos. 27-134 of SEQ ID NO: 1, in case of 10 human BNP. Accordingly, in a preferred embodiment of the present invention, a special attention is preferably paid in the selection of an antigen to obtain an antibody capable of recognizing the amino acid sequence shown by amino acid Nos. 27-102. The preparation of such an antibody can be carried out by any one of methods known in the art.

15 Theoretically,  $\gamma$ -BNP molecule can be cleaved by protease at sites corresponding to No. 47 (Arg), No. 53 (Lys) and No. 72 (Arg) in the amino acid sequence of SEQ ID NO: 1, and, therefore, an antibody recognizing an amino acid sequence shown by amino acid Nos. 73-102 of SEQ ID NO: 1 can be used as the second antibody.

20 The assay of the present invention can be either a competitive- or sandwich-assay and an antibody to be used may be a monoclonal- or polyclonal-antibody.

At least one of the first and the second antibodies may be labeled detectably or immobilized on a solid support.

25 The method for labeling or immobilizing an antibody is known to one ordinary skilled in the art. Examples of label include without limitation radioactive isotopes, enzymes, fluorescent substances, luminescent substances, and particles. The labeling of an antibody can be carried out according to a method known to one ordinary

skilled in the art, for example, that described by Kono et al. (Kaku-Igaku Gijutsu, 13(1), 2, (1993)).

The present invention further provides a kit for immunoassay specific to mammalian  $\gamma$ -BNP derivatives, characterized in that it  
5 comprises two antibodies wherein the first antibody is reactive with mammalian  $\alpha$ -BNP and the second antibody is reactive with mammalian prepro-BNP or  $\gamma$ -BNP derivatives and is not reactive with  $\alpha$ -BNP.

The kit of the present invention can be for a competitive- or  
10 sandwich-assay and an antibody to be used may be a monoclonal- or polyclonal-antibody.

At least one of the first and the second antibodies may be labeled detectably or immobilized on a solid support. The kit of the present invention may further contain a means for detecting the label.  
15 Examples of label include without limitation radioactive isotopes, enzymes, fluorescent substances, luminescent substances, or particles.

The following examples and test examples are provided to further illustrate the present invention, without limiting the scope  
20 thereof.

#### Example 1

##### Measurement of $\gamma$ -BNP Derivatives by Sandwich IRMA

Throughout the following Examples, the ordinary reagents used are of special grade supplied by Wako Pure Chemicals Industries,  
25 Ltd. or Nacalai Tesque, Inc. The bovine serum albumin (BSA) was purchased from Sigma.

###### (1) Preparation of Plasma Sample

1) Venous blood was collected from patients of cardiac disease or healthy volunteers and placed in blood-collecting tubes

containing EDTA and aprotinin (500 KIU/l, Sigma) derived from bovine lung. The tubes were centrifuged (x2000 g at 4°C) for 5 minutes with H-107RGA (Kokusan) to separate blood cells. The resultant plasma samples were freezed and stored at -80°C until use.

5           2) The plasma samples prepared in 1) above from patients of cardiac disease or healthy volunteers were fractionated by gel filtration HPLC system LC10A (Shimadzu) equipped with Superdex 75 10/30 column (Pharmacia). After equilibrating the column with 0.1 M phosphate buffer (pH 7.5, 0.3M NaCl, 5 mM EDTA) at flow rate  
10 of 1 ml/min, 1 ml of plasma sample was injected and 1 ml each of effluent eluted from the column was collected. Each fraction was subjected to the measurement by assay systems for measuring  $\alpha$ -BNP or  $\gamma$ -BNP as described in (2)-2) and (2)-3) below, respectively.

(2)         Construction of Assay System For Measuring  $\alpha$ -BNP- or  $\gamma$ -BNP Derivative

1)         In the assay system, the following peptides, antibodies and kits were used.

- Human  $\alpha$ -BNP (Peptide Institute)
- Antibody against the amino terminal region of  $\gamma$ -hBNP (amino acid Nos. 27-64 of SEQ ID NO: 1) (Peptide Institute)
  - Monoclonal antibody against the carboxy terminal structure of  $\alpha$ -BNP (BC203). BC203 is an immobilized antibody appended to SIONORIA BNP kit (Shionogi), wherein a monoclonal antibody directed to the carboxy terminal structure of  $\alpha$ -BNP is immobilized on beads.
    - Monoclonal antibody against the ring structure of  $\alpha$ -BNP (KYBNPII). KYBNPII is a monoclonal antibody appended to the SIONORIA BNP kit (Shionogi), which is directed to the ring structure (112-128) of  $\alpha$ -BNP, and is labeled with  $^{125}\text{I}$ .

2) Measurement of Plasma Fraction by Assay System for  
 $\alpha$ -BNP

The measurement of  $\alpha$ -BNP was carried out by commercially available "SHIONORIA BNP kit" (Shionogi). The assay is based on 5 sandwich IRMA (Immunoradiometric Assay) which uses a monoclonal antibody KYBNPII specific for the ring-structure of  $\alpha$ -BNP and another monoclonal antibody BC203 specific for the carboxy terminal structure of  $\alpha$ -BNP. The assay was carried out in accordance with the supplier's instructions.

10 That is, 100  $\mu$ l each of samples to be assayed or standard solutions (0, 4, 10, 150, 600 or 2000 pg/ml of  $\alpha$ -BNP solution) were dispensed into a polystyrene test tube. To the test tube was added 200  $\mu$ l of iodine-labeled anti-BNP antibody ( $^{125}$ I) solution, followed by one polystyrene bead on which anti-BC203 antibody has been 15 immobilized. The mixture was stirred and allowed to react by leaving stand for 18 hours at 4 °C. After washing twice with 2 ml of washing solution, radioactivity was measured on  $\gamma$ -counter ARC-600 (Aloka)

The results are shown in Figs. 1 and 2.

3) Measurement of Plasma Fraction by Assay System  
20 For  $\gamma$ -BNP Derivative

An antibody against amino terminal portion (Nos. 27-64) of  $\gamma$ -hBNP was first labeled with  $^{125}$ I.

IgG was purified from anti-serum (Peptide Institute) raised against amino terminal portion (amino acid Nos. 27-64 of SEQ ID NO: 25 1) of  $\gamma$ -hBNP using MASPII kit (Bio-Rad) and displaced with 0.5 M phosphate buffer (pH 7.5) using Centricon 30 (Amicon). The labeling of antibody was carried out by the chloramine T method. To a glass tube was dispensed 170  $\mu$ l of purified IgG solution (77.6  $\mu$ g, IgG), and 10  $\mu$ l of Na $^{125}$ I solution (34.2 MBq, Amersham) was added. After

addition of 0.1 % chloramine T solution ( $20 \mu l$ ), the mixture was vigorously stirred at room temperature for 30 seconds. The reaction was quenched by adding  $20 \mu l$  of 0.25 % sodium pyrosulfite solution and  $20 \mu l$  of 5% aqueous potassium iodide solution. When the 5 reaction mixture was treated with Ampure SA column (Amersham) to remove unreacted  $^{125}I$  and to desalt, solution containing  $^{125}I$ -labeled antibody was obtained.

The sandwich IRMA was then carried out in plasma fractions by using the resultant antibody and polystyrene beads on which an 10 antibody recognizing the carboxy terminal structure of  $\alpha$ -BNP (BC203).

To a polystyrene tube was placed  $100 \mu l$  each of samples to be assayed, followed by  $200 \mu l$  of 0.1 M phosphate buffer (pH 7.5, 0.3M, 5 mM EDTA, 0.2% BSA and 500 KIU/l bovine lung aprotinin 15 (Sigma)) and one polystyrene bead on which BC203 antibody has been immobilized. The mixture was stirred and allowed to react by leaving stand for 18 hours at  $4^{\circ}C$ . After washing twice with 2 ml of washing solution,  $300 \mu l$  of  $^{125}I$ -labeled antibody solution was added. The mixture was stirred and allowed to react by leaving stand for 18 hours 20 at  $4^{\circ}C$ . After washing twice with 2 ml of washing solution, radioactivity was measured on  $\gamma$ -counter ARC-600 (Aloka). The results are shown in Fig. 3.

### (3) Results

Figs. 1, 2 and 3 show the chromatograms of gel filtration 25 HPLC of plasma samples obtained from patients, wherein A is the position of elution of  $\alpha$ -BNP.

Fig. 1 shows the result of the measurement conducted by the  $\alpha$ -BNP assay kit described in (2)-2) above. In the Fig. 1, the vertical axis represents the concentration of BNP-like substances in each

fraction and the horizontal axis the volume of effluent eluted from the column as measured by SHINORIA BNP kit. The solid triangle, open square, and open rhombus respectively represent the measurements in different plasma samples.

5 Fig.2 shows the result of the measurement conducted by the  $\alpha$ -BNP assay kit described in (2)-2) above in samples different from those shown in Fig. 1. In the Fig. 2, the vertical axis represents the concentration of BNP-like substances in each fraction and the horizontal axis the volume of effluent eluted from the column as  
10 measured by SHINORIA BNP kit. The solid triangle and solid square respectively represent the measurements in different plasma samples.

From Figs. 1 and 2, it is revealed that there exist substances of molecular weight larger than  $\alpha$ -BNP and having BNP-like immunoreactivity in the plasma of patients of cardiac disease, and  
15 that they are the major substances having BNP immunoreactivity.

Fig.3 shows the results of the measurement conducted by  $\gamma$ -BNP assay kit described in (2)-3) above in the same samples those shown in Fig. 2. In Fig. 3, the vertical axis represents the radioactivity measured by the  $\gamma$ -BNP immunoassay system and the  
20 horizontal axis the volume of effluent eluted from the column. The solid circle represents the measurements of  $\alpha$ -BNP, which obtained after fractionating human  $\alpha$ -BNP solution by HPLC in a similar manner as described in the case of plasma.

From Fig. 3, it is revealed that the immunoassay specific for  
25  $\gamma$ -BNP derivative of the present invention can detect the major substances with BNP immunoreactivity, but cannot  $\alpha$ -BNP at all.

The results above indicate that the immunoassay for  $\gamma$ -BNP of the present invention is insensitive to  $\alpha$ -BNP but specific to  $\gamma$ -BNP derivatives. Further, it has also been revealed that  $\gamma$ -BNP is

the major substance having BNP immunoreactivity.

#### Test Example 1

##### Stability of $\gamma$ -BNP Derivatives and $\alpha$ -BNP in Plasma

Fractions suspected to contain  $\gamma$ -BNP derivative were  
5 collected from those obtained by treating plasma samples collected  
from patients of cardiac disease by gel filtration HPLC. Venous blood  
was collected from healthy volunteers using blood-collecting tubes  
containing EDTA in the absence of bovine lung aprotinin. Plasma  
samples (the minimum detection limit of  $\alpha$ -BNP < 4 pg/ml) were  
10 prepared in a manner similar to that described in (1)-1) above. The  
plasma sample was allowed to stand for 0, 2, 6, 24 hours at room  
temperature (25°C) after addition of the fraction. The stability of BNP  
derivative was evaluated by determining the BNP immunoreactivity in  
the plasma sample by means of SHIONORIA BNP kit for assaying  $\alpha$ -  
15 BNP.

Separately, the stability of  $\alpha$ -BNP was evaluated using a  
plasma sample prepared by adding chemically synthesized  $\alpha$ -BNP to  
plasma collected from healthy volunteers and standing for 0, 2, 6 and  
24 hours at 4°C in the absence of bovine lung aprotinin as described  
20 above. The BNP immunoreactivity in the plasma sample was  
determined by SHIONORIA BNP kit in the same manner as above.

The stability of  $\gamma$ -BNP derivatives and  $\alpha$ -BNP in plasma  
samples are shown in Figs. 4 and 5, respectively.

From Fig. 4, it is revealed that  $\gamma$ -BNP derivatives do not lose  
25 significantly the immunoreactivity compared with the initial activity  
even after 24-hour-standing at 25°C. From Fig. 5, by contrast, it is  
revealed that  $\alpha$ -BNP loses the immunoreactivity to about 40% based  
on the initial activity after 24-hour-standing at 4°C.

The above results demonstrate that  $\alpha$ -BNP is far less stable

compared with  $\gamma$ -BNP derivative in blood and that the latter is much more suited in the diagnosis of cardiac diseases than the former.

## INDUSTRIAL APPLICABILITY

As mentioned above, the BNP level in patients of heart failure sometimes increases to several tens times to several hundreds times of that of healthy normal subjects, and the change of BNP in the cases of heart failure is so marked that no other hormones are incomparable therewith. For this reason, the usefulness of BNP measurement has been proposed.

The immunoassay of the present invention allows to determine specifically  $\gamma$ -BNP derivatives without measuring  $\alpha$ -BNP.

Accordingly, the present immunoassay can be a clinically significant means for diagnosis and prognostic monitoring of heart failure, which leads to conclusion/judgment somehow different from those resulted from conventional BNP assay.

Further, it is herein disclosed for the first time that  $\gamma$ -BNP, which is a target substance to be assayed by the present method, is stable in blood. Therefore, immunoassay of the present invention provides stable and reliable clinical data without being affected by the process of collecting or storing samples, or the time from the collection until measurement. Further, the immunoassay of the present invention does not require any special pretreatments of blood sample and therefore gives clinical data conveniently, thereby contributing to the establishment of highly accurate diagnosis of cardiac diseases.